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THE NATURE OF THE ESTROGENIC
SUBSTANCE IN HUMAN MALE
URINE AND BULL TESTIS

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY AND PHARMACOLOGY

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THE NATURE OF THE ESTROGENIC SUBSTANCE IN HUMAN MALE URINE AND BULL TESTIS*†

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The fact that male urine and testis tissue contain estrogenic activity has been known for some time. Fee, Marrian, Parkes (1), Glimm and Wadehn (2), Lacqueur and de Jongh (3), Zondek (4, 5), and Loewe (6) have reported the presence of estrogenic substance in male urines. Dodds, Greenwood, and Gallimore (7), Laqueur and de Jongh (3), and Brouha and Simmonet (8) have reported estrogenic activity in testis.

Most of the workers have confined themselves to a qualitative study by means of the reaction on spayed mice or rats. Fee, Marrian, and Parkes have suggested that the active material is identical with the female sex hormone "estrin" on the basis of the marked uterine hypertrophy observed in spayed female rats and the positive vaginal smear reaction. Zondek§ makes the assertion that the estrogenic substance in stallion urine is similar to the follicular hormone on the basis of the production of uterine hypertrophy, an antimasculine action, the induction of lactation in the male guinea pig, and the positive vaginal smear reaction. However, it must be remembered that estrus and uterine hypertrophy are biological reactions produced in greater or less degree by the two female hormones thus far isolated. It has also been shown by Cook, Dodds, Hewett, and Lawson (9) that certain synthetic phenanthrene and dibenzanthracene derivatives are also capable of producing estrus and uterine hypertrophy. Therefore, we feel that a quantitative biological study is necessary to differentiate among theelin, theelol, and any other possible estrogenic substance.

In this communication we have undertaken to characterize this estrogenic activity in human male urine and bull testis by comparative quantitative biological studies on these preparations and on the crystalline female sex hormones, theelin and theelol.

For the urinary preparations, 110 liters of normal male urine were collected from males ranging in age from 20 to 30 years. Immediately after collection, the urine was acidified with HCl to approximately 1 per cent by volume, the urine was extracted four times with benzene in a continuous extractor already described by Gallagher and Koch (10). The

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§After this paper had been presented, Deulofeu and Ferrari (15) reported that they obtained crystalline estrus-producing hormone from stallion urine.

benzene solution was evaporated to dryness, taken up in ethyl ether, and shaken with aqueous sodium bicarbonate solution, and finally with water. The ether solution was evaporated to dryness, yielding a viscous red brown oil which was made up to volume in 95 per cent ethanol. This total benzene extract will be referred to as UD II total extract. A measured portion of the alcohol solution, UD II total extract, was evaporated to dryness, taken up in 200 cc. of ethyl ether, and extracted 25 times with 100 cc. portions of fresh 10 per cent sodium hydroxide solution. The watery alkaline extracts were combined and made acid by addition of an excess of 10 per cent sulphuric acid. The acid solution was extracted with ethyl ether. The ether was distilled and the remaining brown oil was made up to volume in 95 per cent ethanol. This fraction will be known as UD II alkali-soluble. The ether solution containing the alkali insoluble fraction was evaporated to dryness and the reddish oily residue, made up to volume in 95 per cent ethanol, will be referred to as UD II alkali-insoluble.

The theelin assayed 7630 international rat units per mgm. from which it might be inferred that our preparation is slightly inferior to the international standard. The theelol assayed 125 international rat units per mgm., which compares favorably with the values of Butenandt and Browne (16) by essentially the same method of assay as we used, namely, one injection of theelol dissolved in oil.

The theelin and theelol employed in this study were obtained from pregnancy urine and were the crystalline compounds.

The urine fractions, the bull testis preparations, the theelin, and the theelol were assayed on adult spayed female rats according to the method described by D'Amour and Gustavson (11). Twenty-five animals were used for the assay of each preparation and the results referred to a standard which was assayed at the same time. The values given for the estrogenic activity in the charts and graphs are our own rat unit values which are seven times the international rat unit, that is: 1 RU=7 IRU. The male hormone content was determined by the comb growth method published by Gallagher and Koch (12, 13).

Infantile female albino rats from our own stock colony were employed in this study. Injections of the active material in 0.2 cc. of olive oil were started on the 25th day of life and were continued once a day for five days. All values of active substance in the following charts and graphs are expressed in dosage per day. During the injection period the animals were carefully observed each day for vaginal introitus. The animals were killed on the 30th day. Body, ovarian, and uterine weights were determined. Litter mates were used in all of the comparative studies.

Statistical analysis of the data seemed unnecessary in view of the relatively small individual variations and reproducibility of our results. The greatest individual variation encountered occurred in the group of five animals injected with 0.1 RU of theelol where one animal showed a uterine ratio deviating from the mean by 33 per cent. On the average no one

animal in a group of five showed a greater deviation than 20 per cent from the mean uterine ratio, and the majority of animals in the group showed a deviation from the mean of less than 10 per cent. In a group of five animals injected with 0.1 rat unit of theelin we found a mean uterine ratio of 0.751. To test the reproducibility of our results, another group of five animals was injected under the same conditions. This time we recorded a mean uterine ratio of 0.797, giving us a deviation of 5 per cent from the first observation. So also on 0.3 rat unit of theelin, the mean

TABLE I

Estrogenic Activity R. U.	No. of Animals on Each Preparation	Mean Uterine Weights in Mgm.			
		Theelin	Theelol	UD II Alkali Soluble	UD II Total Extract
0.01	5	20.4	35.7		20.7
0.10	5	36.8	49.0	43.8*	66.9†
0.20	5	53.2	40.2	57.3	83.9
0.30	5	82.9	51.7	99.3	123.
0.55	3	206.0			
1.0	3		85.5		
1.5	3		70.5		

*0.12 rat unit. †0.11 rat unit. On a given dose the comparison of the four preparations was made on litter mates.

uterine ratios were 1.78 and 1.60 on two groups of five animals each. It is evident, therefore, that our values are significant and reproducible. It is likewise apparent that when four preparations are compared as for example in Tables I, II, III and IV, the significance of the results is increased by the use of a littermate animal on each preparation, as we have done throughout.

TABLE II

Estrogenic Activity R. U.	No. of Animals on Each Preparation	Ratio = $\frac{\text{Mgm. of Uterus}}{\text{Gm. of Body Weight Less Gut}}$			
		Theelin	Theelol	UD II Alkali Soluble	UD II Total Extract
0.01	5	0.499	0.862		0.519
0.10	5	0.751	1.059	0.893*	1.56†
0.20	5	0.978	0.819	1.013	1.75
0.30	5	1.78	1.10	2.07	2.53
0.55	3	5.1			
1.0	3		1.7		
1.5	3		1.4		

*0.12 rat unit. †0.11 rat unit.

Tables I, II, and III present a summary of the uterine and vaginal responses to various levels of estrogenic activity of the urinary preparations compared to theelin and theelol. In Table I we have the response expressed in mgm. of uterine weight, in Table II, the ratio of mgm. of uterus to gm. of body weight less gut, and in Table III, vaginal introitus in per cent.

TABLE III

Estrogenic Activity R. U.	No. of Animals on Each Preparation	Vaginal Introitus in Per Cent			
		Theelin	Theelol	UD II Alkali Soluble	UD II Total Extract
0.01	5	0	60		0
0.10	5	0	100	0*	70‡
0.20	3	30	100	20	100
0.30	5	100	100	100	100
0.55	3	100			
1.0	3		100		
1.5	3		100		

*0.12 rat unit. ‡0.11 rat unit.

We find that in doses of 0.01 rat unit, expressed in terms of estrogenic activity, the estrogenic substance shows a similarity in vaginal introitus and uterine weight reactions to theelin but quite different from theelol. At 0.1 rat unit the estrogenic substance in the total benzene extract (UD II total extract) does not resemble theelin alone, but the alkali-soluble fraction (UD II alkali-soluble) from the total extract closely approximates theelin on both the vaginal introitus and uterine weight reactions. The alkali-insoluble fraction (UD II alkali-insoluble) when injected in a concentration equivalent to the volume of urine represented in 0.1 rat unit of UD II total extract gives no response on the uterus or vagina. When

TABLE IV

UTERINE HYPERTROPHY AND VAGINAL INTROITUS

Responses Produced by Different Levels of the Alkali-Insoluble Fraction from Normal Male Urine Compared to Untreated Normals

Daily Dosage of UD II Alkali- Insoluble		Number of Animals Used	Uterus	Body Weight Less Gut	Ratio: Uterus	Vaginal Introitus
Liters of Urine	B. U.				Body Weight Less Gut	
			Mgm.	Gm.		Per Cent
0.0	0.0	18	23.4	40.8	0.540	0
0.014	0.75	5	27.4	51.8	0.537	0
0.385	20.6	3	58.1	52.1	1.092	100
1.32	70.6	2	102.6	44.4	2.40	100

UD II alkali-insoluble and UD II alkali-soluble are recombined and administered in identical dosage, they give a response equivalent to UD II total extract on both the uterus and the vagina. From this we postulate a substance present in UD II alkali-insoluble which exerts an enhancing effect on the vagina and uterus when combined with theelin. Increasing the estrogenic level to 0.2 and 0.3 rat unit we find essentially confirmation of our results at the lower levels. At 0.3 rat unit the small discrepancy between theelin and UD II alkali-soluble probably is due to small amounts of the enhancing substance mechanically introduced into the alkali solution during the 25 extractions with 10 per cent sodium hydroxide.

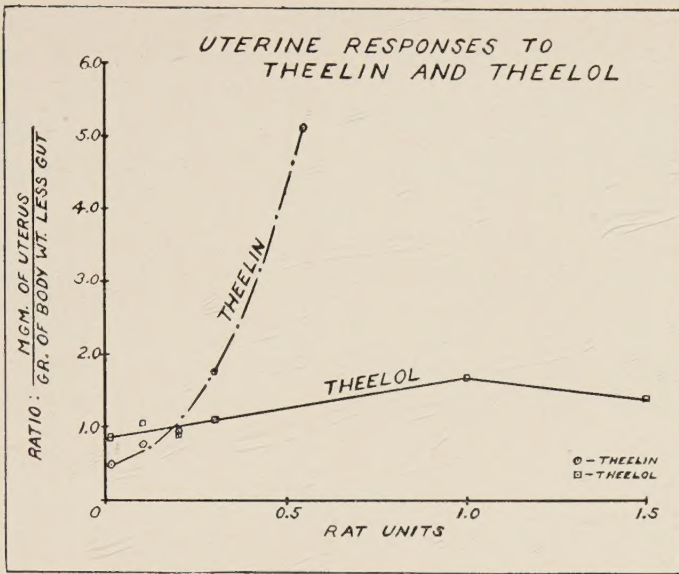


FIGURE 1

In Figure 1 we see the characteristic curves of uterine hypertrophy responses for theelin and theelol. We see at a glance the striking difference between the two female sex hormones. Although theelol gives a positive response on the uterine hypertrophy reaction at a lower rat unit level, theelin is more efficient and produces a more marked effect with increase in estrogenic activity. This peculiar difference in quantitative response to increasing doses of rat units supplied in the form of theelin and theelol obviously gives us a very good biological method by means of which to

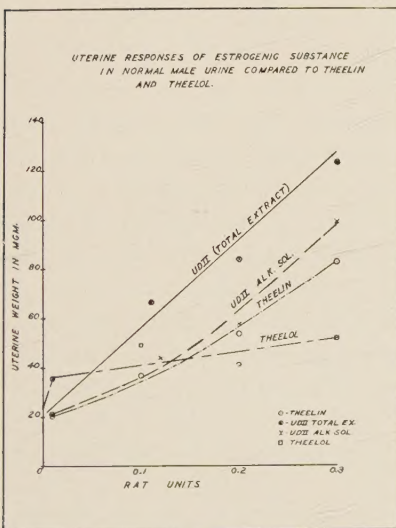


FIGURE 2

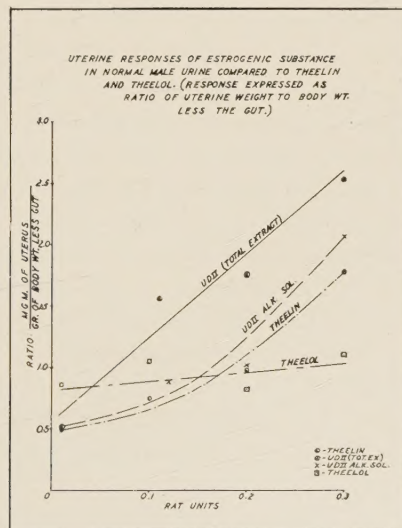


FIGURE 3

distinguish between these two substances in various biological preparations. The data presented in this paper confirm the value of this method of approach.

In Figures 2 and 3 we have composite curves of UD II total extract, UD II alkali-soluble, theelin, and theelol. In Figure 2 uterine hypertrophy is expressed in mgm. while in Figure 3 it is expressed in the ratio of mgm. of uterine weight to gm. of body weight less the gut. These graphs summarize the data in the tables, I, II, and III, and also show that we get the same significant results whether the response is expressed in weight of uterus or in ratio of uterus to body weight. By comparing the curves for UD II alkali-soluble and theelin, we observe a very striking similarity whereas theelol and UD II total extract do not agree with each other and also do not agree with theelin. This clearly shows first, that the

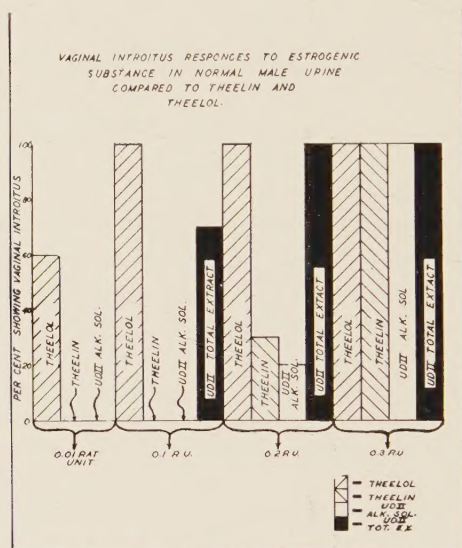


FIGURE 4

estrogenic activity extractable from the urinary male hormone by aqueous sodium hydroxide is theelin and not theelol and, second, that a uterine-enhancing substance other than theelin and theelol is present in the total urinary extract. It is interesting to note that if we compare the vaginal introitus activity of theelin and theelol on a weight basis we find theelin to be the more active. This observation is contrary to that of Curtis and Doisy (14), who, however, employed a different technique than we did.

Figure 4 is a summary of the data dealing with vaginal introitus. It brings out essentially the facts that on a rat-unit basis theelol is more active than theelin, that UD II alkali-soluble is strictly comparable to theelin, and that UD II total extract is more active on the vagina than UD II alkali-soluble.

In view of the marked difference in the uterine hypertrophy and vaginal introitus reactions of the total extract (UD total extract) and the

alkali-soluble extract (UD II alkali-soluble) it became extremely interesting to investigate the alkali-insoluble fraction (UD II alkali-insoluble).

Table IV gives a summary of the responses obtained at three levels of UD II alkali-insoluble compared to uninjected normals of the same age. At a level equivalent to 0.014 liter of urine we see no effect on uterus or vagina, but at 0.385 liter and 1.32 liters of urine we observed a distinct and definite reaction.

The question now arises as to the material in UD II alkali-insoluble responsible for this uterine hypertrophy and vaginal introitus. What are the possibilities? We are led to consider the four following possibilities: first, the remote possibility of theelol present in UD II alkali-insoluble; second, theelin alone; third, male hormone or some undescribed substance; fourth, theelin plus enhancing substance.

At the present time we cannot present a complete solution of this problem but merely indicate our observations which no doubt throw some light on this complicated system. As to the first mentioned possibility we may say that it is eliminated because it is very improbable that theelol, being more soluble in aqueous alkali than theelin, would not be extracted after 25 extractions with 10 per cent sodium hydroxide. Further, by calculations from the theelol curve we find that since the dosage equivalent to 0.385 liter of urine of UD II alkali-insoluble gives us a uterine ratio of 1.09, the highest dosage 1.32 liters of urine should give a uterine ratio of 1.69 if the typical theelol reaction were obtained. However, we actually find 2.40 (see Figure 1). The second possibility is promptly ruled out by similar calculations from the pure theelin curve. From the curve in Figure 1 we should get a ratio above 5 for the highest dilution where we find 2.4. The third possibility, that the action is due to male hormone or some undescribed substance, is under investigation at the present time. It is, of course, possible that the male hormone may be the enhancing substance, thus presenting a possible physiological rôle for the male hormone found in the urine of women. This would parallel the observation of Freud (17) that estrogenic substances play an important part in the response of the mammalian accessory reproductive organs to the male hormone. The fourth possibility, that is, theelin plus enhancing substance, has so far given us some encouraging results. We have tested our values on the UD II total extract curve since this curve represents theelin plus enhancing substance. At 0.385 liter of urine we find a uterine ratio of 1.09 which corresponds to 0.08 rat unit of the UD II total extract. At 1.32 liters, which is 3.4 times the smaller concentration, we should expect the effect from 0.27 rat unit or a uterine ratio of 2.38 (see Table IV and Figure 1). We actually find 2.40, but since the values have been determined on a limited number of animals, too much significance should not be placed on the values.

By a method similar to that employed for the work outlined above on the urine preparations, it has been found by us that the alkali-soluble frac-

tion from the total extract of bull testis contains an estrogenic substance similar to theelin on the basis of vaginal introitus and uterine hypertrophy.

SUMMARY

We have compared the uterine hypertrophy and vaginal introitus responses of equivalent rat unit dosages of theelin, theelol, and estrogenic substance from male urine. These observations were made at estrogenic levels ranging from 0.01 rat unit to 0.3 rat unit. Our method involved the daily administration of the active material in olive oil to 25-day-old female albino rats for five days, and observing the uterus and vagina on the sixth day. At each rat unit level for each preparation five animals were used except in a few cases where three animals were deemed sufficient. Litter mates were used throughout the comparative studies. A total of 161 animals were used for the comparative studies.

The quantitative data presented lead to the following conclusions:

1. There is a distinct difference between theelin and theelol in their respective abilities to cause hypertrophy of the uterus and cause vaginal introitus. On an estrus-rat-unit basis, theelol is more effective in causing vaginal introitus as originally demonstrated by Curtis and Doisy (14), whereas theelin produces an enormously greater uterine hypertrophy.

2. The estrogenic substance in the total benzene extract from normal male urine resembles neither theelin nor theelol in causing either vaginal introitus or uterine hypertrophy.

3. The estrogenic substance in the alkali-soluble fraction of the total benzene extract is biologically identical with theelin. It is not comparable to theelol in causing either vaginal introitus or uterine hypertrophy.

4. The evidence presented points to the presence of a substance in the alkali-insoluble fraction of the total extract which, when administered with theelin, enhances the action on both the vagina and the uterus. This substance is neither theelin nor theelol.

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